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Attraction of pepper weevil to volatiles from damaged pepper plants

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Abstract

Pioneer herbivorous insects may find their host plants through a combination of visual and constitutive host-plant volatile cues, but once a site has been colonized, feeding damage changes the quantity and quality of plant volatiles released, potentially altering the behavior of conspecifics who detect them. Previous work on the pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae), demonstrated that this insect can detect and orient to constitutive host plant volatiles released from pepper [*Capsicum annuum* L. (Solanaceae)]. Here we investigated the response of the weevil to whole plants and headspace collections of plants damaged by conspecifics. Mated weevils preferred damaged flowering as well as damaged fruiting plants over undamaged plants in a Y-tube olfactometer. They also preferred volatiles from flowering and fruiting plants with actively feeding weevils over plants with old feeding damage. Both sexes preferred volatiles from fruiting plants with actively feeding weevils over flowering plants with actively feeding weevils. Females preferred plants with 48 h of prior feeding damage over plants subjected to weevil feeding for only 1 h, whereas males showed no preference. When attraction to male- and female-inflicted feeding damage was compared in the Y-tube, males and females showed no significant preference. Wind tunnel plant assays and four-choice olfactometer assays using headspace volatiles confirmed the attraction of weevils to active feeding damage on fruiting plants. In a final four-choice olfactometer assay using headspace collections, we tested the attraction of mated males and virgin and mated females to male and female feeding damage. In these headspace volatile assays, mated females again showed no preference for male feeding; however, virgin females and males preferred the headspace volatiles of plants fed on by males, which contained the male aggregation pheromone in addition to plant volatiles. The potential for using plant volatile lures to improve pepper weevil monitoring and management is discussed.

Introduction

Plants respond to insect herbivory by altering the amount and type of volatile compounds they synthesize and release. Depending on the plant species or type of herbivore causing the damage, quantitative or qualitative changes in the volatile plume may be observed (reviewed by Paré & Tumlinson, 1999). Changes in host plant volatiles have been shown to convey information related to the location of hosts (Kalberer et al., 2001), the location of aggregations (Loughrin et al., 1996) and amount of competition present in a given patch (Meiners et al., 2005). These host-derived volatiles may also act to induce or improve the effectiveness of insect-produced pheromones (reviewed by Landolt & Phillips, 1997; Reddy & Guerrero, 2004).

The response of pest weevils to host plant volatiles has been the focus of several studies. A significant body of research exists on boll weevil (*Anthonomus grandis* Boheman) response to host plant volatiles. Behavioral and electrophysiological recordings demonstrated attraction to cotton-specific and green leaf volatiles (Dickens, 1984, 1986, 1989; Dickens & Moorman, 1990; Dickens et al.,

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1990). Field tests with the aggregation pheromone, grandlure, paired with the green leaf volatiles, *trans*-2-hexen-l-ol, *cis*-3-hexen-l-ol, or l-hexanol, increased the number of weevils captured in traps when compared to grandlure alone (Hedin et al., 1979). Although attraction of the boll weevil to specific-cotton and green leaf volatiles has been verified, to our knowledge, the effect of induced cotton volatiles after feeding damage has not been investigated. Attraction to constitutive and/or induced host plant volatiles has also been demonstrated in other members of the genus *Anthonomus*, including the cranberry weevil, *A. musculus* Say (Mechaber, 1992; Szendrei et al., 2009), strawberry blossom weevil, *A. rubi* Herbst (Bichao et al., 2005a,b) and apple blossom weevil, *A. pomorum* L. (Kalinova et al., 2000).

The pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae), is a common pest of cultivated pepper (*Capsicum* spp.) in the southern United States, Central America, and the Caribbean region. Feeding and oviposition by weevils causes abscission of the flowers and fruit and lowers crop yields with estimates as high as 100% loss (Genung & Ozaki, 1972). The weevil can also survive and reproduce in wild nightshade plants (*Solanum* spp.) found around fields when pepper is not in production. The weevil spends the fallow season in weeds and then moves back into the fields when the next crop is planted (Patrock & Schuster, 1987). The ability of the pepper weevil to move from cultivated fields to surrounding nightshades during crop-free periods and back again stimulated our interest in the potential use of volatile cues by pepper weevils during host plant location. Previously, pepper weevil attraction to constitutive volatiles from host and non-host plants was evaluated (Addesso & McAuslane, 2009). Volatiles from undamaged pepper plants and fruit attracted both male and female weevils. In choice tests, females also showed a consistent preference for pepper plant volatiles over American black nightshade (*Solanum americanum* Mill.).

Here we continue our investigation of host plant attraction of the pepper weevil, specifically to explore the attraction by volatiles induced by conspecific feeding damage. To this end, behavioral observations were made for weevils in a Y-tube using flowering and fruiting jalapeño plants [*Capsicum annuum* L. (Solanaceae)] as well as excised jalapeño fruit. Assays were also performed in a wind tunnel to determine if weevils would respond with similar upwind movement to a more distant and dispersed volatile source. Additionally, headspace volatiles from the most attractive plant treatments were collected on adsorption filters and assayed in a four-choice olfactometer as a first step towards identifying the most attractive blend of volatile to use in future studies of semiochemical management strategies.

Materials and methods

Insects and plants

Pepper weevil, *A. eugenii*, came from a colony maintained at the University of Florida in Gainesville, FL, USA. The colony was established in 2004 from weevils collected in southern Florida near the city of Clewiston (26°45′N, 80°56′W) and supplemented with field-caught insects annually. Insects were maintained under a L14:D10 regime at 27 °C and 30% r.h. The colony was maintained on jalapeño peppers (*C. annuum*) grown at the University of Florida, with water and honey supplements.

Jalapeño pepper plants were grown from seed (USDA certified organic, item #46508; Southern Exposure Seed Exchange, Mineral, VA, USA) in 12-cm-square pots in a 50/50 ratio of Metro-Mix 200 and 500 (SunGro, Bellevue, WA, USA) at the USDA-ARS-CMAVE laboratory in Gainesville, FL. Plants were watered as needed and fertilized using Osmocote® 14-14-14 slow release pellets (The Scotts Company, Marysville, OH, USA). Plants used in these assays were approximately 8 or 10 weeks old at the flowering and fruiting stage, respectively.

Y-tube bioassays

Bioassays were conducted in a glass Y-tube olfactometer (12 cm common tube, 10 cm arms, and 2.5 cm internal diameter; Analytical Research Systems, Gainesville, FL, USA) using the methods of Addesso & McAuslane (2009). Ten-day-old weevils were sexed (Eller, 1995) and starved for 12 h prior to assay without access to water. All insects were drawn from the colony cage and females were mated. Both sexes were assayed simultaneously in one of the two Y-tubes until a total of 50 males and 50 females had responded. Twenty-five insects of each sex were assayed individually each day over a 3-day period. Each insect was given 15 min to make a choice of arms in the olfactometer. Weevils that passed halfway or further into one arm of the Y-tube were recorded as making a choice. If no choice was made in 15 min, the assay was concluded and the insect was not counted towards the total of 50 responding insects. After half of the insects were assayed for that day, the odor sources were switched to the opposite side to control for right- or left-handed bias. Glassware was rinsed with hot water followed by ethanol and hand-dried between each insect assayed. Assays were run within the previously established activity period for oviposition of 10:00–17:00 hours (Patrock & Schuster, 1992) between 48 and 54 h after feeding treatments were initiated.

In the first set of bioassays, weevils were presented with a choice between volatiles from damaged flowering (8 weeks old; plants with flower buds and open flowers) or fruiting plants (10 weeks old; plants with few open flowers

and immature fruit) and their undamaged controls. For damaged treatments, five female weevils were confined to a branch using an organdy sleeve and twist ties. A piece of cotton was wrapped around the branch beneath the tie to prevent widespread damage or weevils from escaping. The weevils were allowed to feed on the plant for 48 h before use resulting in small puncture wounds (0.5 mm in diameter) in leaf tissue and reproductive structures, and if overly damaged, the abscission of flowers and flower buds. One hour prior to the start of the assay, weevils were removed to give plant wounds time to close. Organdy sleeves were placed on control plants for 48 h, but no weevils were placed inside the bags. Female pepper weevils were used to inflict feeding damage on both 'damaged' and 'active feeding' treatments in all Y-tube, wind tunnel, and four-choice olfactometer experiments to eliminate the influence of male-produced aggregation pheromone typically released when feeding on plant material (Eller et al., 1994; K Addesso, unpubl.). In experiments where males were used to inflict feeding damage, the designations 'male active feeding' and 'female active feeding' were used.

In all Y-tube assays, volatiles from treated branches were presented using custom Tedlar® bags (46 cm × 20 cm; SKC, Eighty Four, PA, USA). Bags were heat-sealed on three sides and contained two polypropylene septa for airflow in and out of the bag. Bags were placed over the treated branches, and like the organdy sleeves, cotton was wrapped around the base of the branch and the bag secured with a zip tie.

In the second set of assays, weevils were presented with a choice between damaged flowering or fruiting plants and plants with actively feeding weevils remaining on the plant. For both treatments, five female weevils were confined to a branch for 48 h using organdy sleeves as previously described. One hour prior to the bioassay, weevils from the damaged treatment were removed, while females continued to feed on the active feeding treatment. In a third bioassay, weevil response to volatiles released from flowering and fruiting pepper plants on which females were actively feeding was compared.

In the fourth assay, fruiting pepper plants with actively feeding females or males were compared to determine whether the sex of the insects causing damage influenced the insect-plant complex's attractiveness. All assay conditions were the same as the previously described active feeding treatment. For the fifth assay, weevils were presented with actively feeding females on fruiting plants with 1 or 48 h of prior feeding damage. In the final assay, weevils were presented with volatiles from undamaged excised pepper fruits and volatiles from fruit with active female weevil feeding. Ten weevils were held in glass chambers on three newly harvested jalapeño fruit (<5 cm long) for 24 h prior to the start of the assay. Weevils were only permitted to feed for 24 h to minimize the decay of excised fruit.

Wind tunnel bioassays

To test whether pepper weevils would respond similarly to plant volatiles at long range, as they had at short distances (as in the Y-tube assays), no-choice bioassays were conducted in four Plexiglas® wind tunnels (120 × 30 × 30 cm) located in a greenhouse (approximate temp 30 °C, 66–75% r.h.) at the USDA-ARS-CMAVE facility in Gainesville, FL as described in Addesso & McAuslane (2009). Weevils were presented with volatiles from whole plants held in Plexiglas chambers (60 cm tall × 15 cm i.d.). Because these chambers required whole plants to be used instead of specific damaged branches, the weevil feeding period was extended to 72 h when plants fed on for shorter time periods failed to elicit a response. Once again, damage consisted of 0.5 mm punctures in fruit, flower buds, and young leaf material as well as some flower and bud drop. Weevils were sexed and held separately in groups of 10 in plastic vials (2.2 cm diameter × 5.0 cm high) with air holes overnight (approximately 15 h) with no food or water prior to assay. The vials were placed into the downwind end of the wind tunnel, 90 cm from the trap. Traps consisted of cylindrical plastic vials (11 cm long × 5 cm in diameter) with a glue board acting as a ramp at an angle of 50° from the floor of the wind tunnel to the trap. Traps were modified in this way due to the pepper weevil's preference for walking upwind rather than flying. Weevils were released into the wind tunnel by removing the vial lid at the start of each assay. Weevil location was categorized and their upwind orientation to pepper volatiles was recorded after 5 h. Total upwind response (>45 cm displacement upwind) and trap contact (contact with the glue board or cylinder trap) were recorded. All experiments were replicated on 4 days for a total of 40 weevils per sex per treatment. Assays were conducted between 72 and 78 h after treatments were initiated. Plant chambers were rinsed with hot water followed by ethanol and hand-dried between each replication. Wind tunnels were wiped down with hot water followed by ethanol and permitted to air dry overnight before the next assay.

In the first wind tunnel assay, weevils were presented with volatiles from an undamaged fruiting pepper plant or volatiles from a pepper plant that had sustained 72 h of feeding damage by female weevils with female weevils removed 1 h prior to bioassay. In the second assay, weevils were presented with either volatiles from a pepper plant with 72 h of feeding damage as in the first assay or volatiles from a pepper plant with 72 h of feeding damage with females still actively feeding. Damage was inflicted in these treatments as described in the Y-tube assays, but in the

wind tunnel assays 4–l Ziploc® vegetable bags (S. C. Johnson & Son, Racine, WI, USA) closed with a twist tie were used to confine the feeding weevils.

Headspace volatile collection, GC/MS analysis of pepper volatiles, and four-choice olfactometer bioassays

Headspace volatile collections of flowering and fruiting jalapeño plants were made using an automated whole plant volatile collection system at the USDA-ARS-CMAVE in Gainesville, FL, USA (Analytical Research Systems). Ten female weevils were confined to flowering (8 weeks old) or fruiting (10 weeks old) pepper plants in the glass volatile collection chambers for 54 h. Volatiles were drawn through a SuperQ-filled cartridge for 6 h (09:00–15:00 hours) beginning after 48 h of weevil feeding. Volatiles from approximately 30 g of pepper fruit were collected after 24 h of feeding by five females, from 09:00 to 15:00 hours. Morning/early afternoon headspace volatiles were analyzed because this was when behavioral bioassays were conducted.

Volatile samples were analyzed directly by cold on-column GC/MS (6890/5975 GC/MS; Agilent, Palo Alto, CA, USA) in EI mode. Samples (1 µl) were injected into a 10-m deactivated retention gap connected to a methyl silicone column (HP5, 30 m × 0.25 mm i.d. × 0.1 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). The injector and column were kept at 30 °C for 5 min and then temperature programmed at 10 °C/min to 260 °C. The He carrier gas flow rate was 30 cm/s (constant flow) and the transfer line temperature was 260 °C. The ion source temperature was 220 °C in EI mode. Spectra library search was performed using a floral scent database compiled at the Department of Chemical Ecology, Göteborg, Sweden, the Adams2 terpenoid/natural product library (Allured Corporation), and the NIST05 library.

Bioassays of headspace volatiles were conducted to verify that the collection process had succeeded in trapping attractive pepper compounds. Additionally, the male feeding assay conducted in the Y-tube was repeated using headspace collections so that samples could be tested for the presence of aggregation pheromone components prior to use in the assay, something that was not possible in the whole plant assays. Bioassays of headspace collections from fruiting plants were conducted in a four-choice olfactometer (Analytical Research Systems) with Teflon tubing connections. The four-choice olfactometer was used because it permitted us to test two treatments at a time alongside a solvent control. Breathing-quality compressed air was pushed through a charcoal filter and humidified with deionized water prior to splitting into four trap chambers. In each of the four volatile chambers a filter paper to which headspace extracts (see below) were applied was placed. Ten insects at a time (male or female) were held 15 h without food or water and placed into the darkened insect inlet beneath the four-choice arena and allowed to climb upward into the center of the assay chamber. Insects were given 30 min to make a choice. A choice was recorded when a weevil walked 10 cm up through the inlet to the arena and a further 11.5 cm toward one of the four arms of the chamber, passing a mark 2 cm in front of the trap tube.

Headspace volatile collections of fruiting jalapeño plants were made using the automated volatile collection system described above. For damage treatments, plants were handled as described in the Y-tube assays. At 48 h post-infestation, all plant treatments were placed in glass guillotine volatile collection chambers. Volatiles were drawn through a SuperQ cartridge for 6 h (09:00–15:00 hours). Individual samples were analyzed by GC-MS to ensure samples contained plant volatile and insect pheromone components. A preliminary bioassay was run to determine the amount of headspace extract required to elicit an upwind response. Based on those results, two collections were pooled by plant treatment for all four-choice bioassays for a total of 12 headspace collection hours in 200 µl of methylene chloride (= 0.6 plant hours/10 µl of headspace extract). Samples were stored at −20 °C until use.

Samples were presented to weevils on a 3.8-cm diameter filter paper cut in half. Twenty µl of mineral oil was applied to the center of both papers. Ten µl of extract (=0.6 plant h equivalents) was applied on top of the mineral oil. Filter papers were left under a ventilation hood for 10 min to allow solvent to evaporate prior to bioassay. In three separate assays, weevils were presented with headspace collections from: (1) undamaged and damaged plants, (2) damaged and active feeding, and (3) active male and active female feeding treatments. The treatment samples were placed in opposite corners of the four-choice olfactometer with solvent/mineral oil controls in the two remaining ports. Ten insects were released in the center of the arena and given 30 min to make a decision. Insects within 2 cm of the trap tube, in the tube, and/or in the trap were recorded as having made a choice. Twelve replications were performed for each sex/treatment combination. In all three assays, 10-day-old mated male and female weevils were tested against the feeding treatments. In the third experiment, virgin 10-day-old females were also tested to determine if mating status had an effect on female response to the combination of plant volatiles and the male-produced aggregation pheromone. Glassware and the olfactometer chamber were rinsed with hot water followed by ethanol and hand-dried between each replication.

Statistical analysis

Behavioral response data were analyzed as percent response using chi-square analysis with an expected probability for Y-tube and wind tunnel assays of 0.5 for each treatment (Proc FREQ, SAS 2006). For the four-choice olfactometer assays, response to the two air ports was pooled for a 0.5 (solvent control), 0.25 (treatment 1), and 0.25 (treatment 2) expected probability.

Results

Orientation of weevils to induced volatiles in a Y-tube

In the first set of assays, male and female pepper weevils preferred damaged flowering plants (male: $\chi^2 = 4.09$, d.f. = 1, P = 0.043; female: $\chi^2 = 8.65$, d.f. = 1, P = 0.0033; Figure 1A) as well as damaged fruiting plants (male: $\chi^2 = 9.68$, d.f. = 1, P = 0.0019; female: $\chi^2 = 12.26$, d.f. = 1, P = 0.0005; Figure 1B) over undamaged controls. In the second set of assays, males and females preferred flowering plants (male: $\chi^2 = 12.76$, d.f. = 1, P = 0.0004; female: $\chi^2 = 16.49$, d.f. = 1, P<0.0001; Figure 2A) as well as fruiting plants (male: $\chi^2 = 13.52$, d.f. = 1, P = 0.0002; female: $\chi^2 = 9.68$, d.f. = 1, P = 0.0019; Figure 2B) with actively feeding weevils over previously damaged plants. In the third assay, males and females preferred fruiting plants with actively feeding weevils over flowering plants with active feeding (male: 84% walked to fruiting active feeding treatment: $\chi^2 = 23.12$, d.f. = 1, P<0.0001; female: 77% walked to fruiting active feeding treatment: $\chi^2 = 15.08$, d.f. = 1, P<0.0001; data not shown). In the fourth assay, males showed no preference between plants with 1 h and 48 h of active female feeding ($\chi^2 = 0.44$, d.f. = 1, P = 0.51), whereas females preferred plants with 48 h of damage (79% walked toward the 48 h treatment: $\chi^2 = 18.29$, d.f. = 1, P<0.0001; data not shown). In the fifth assay, males showed a slight but non-significant preference for active male feeding damage (male: 62% walked to male active feeding: $\chi^2 = 2.88$, d.f. = 1, P = 0.090), but

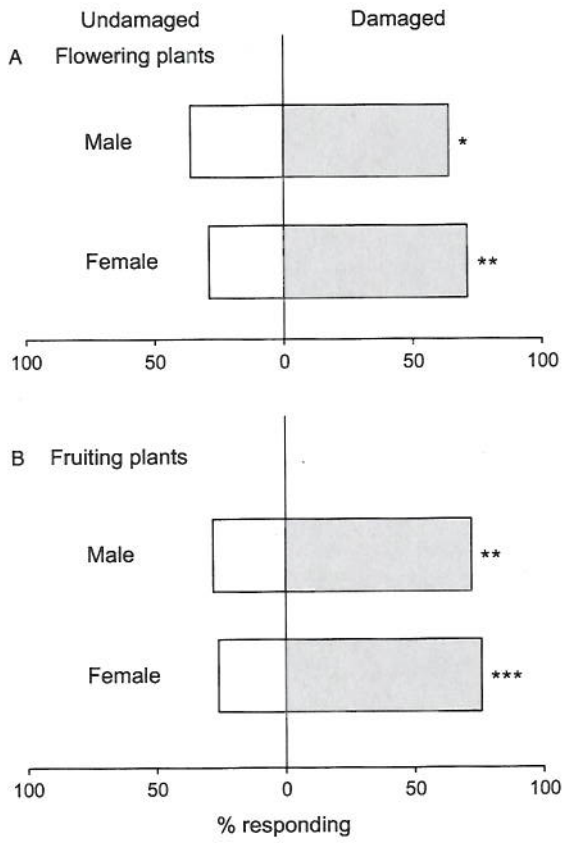

Figure 1 Response of 10-day-old mated *Anthonomus eugenii* to undamaged and female-damaged (A) flowering and (B) fruiting pepper plants in a Y-tube olfactometer. χ^2: *P<0.05, **P<0.01, ***P<0.001; n = 50.

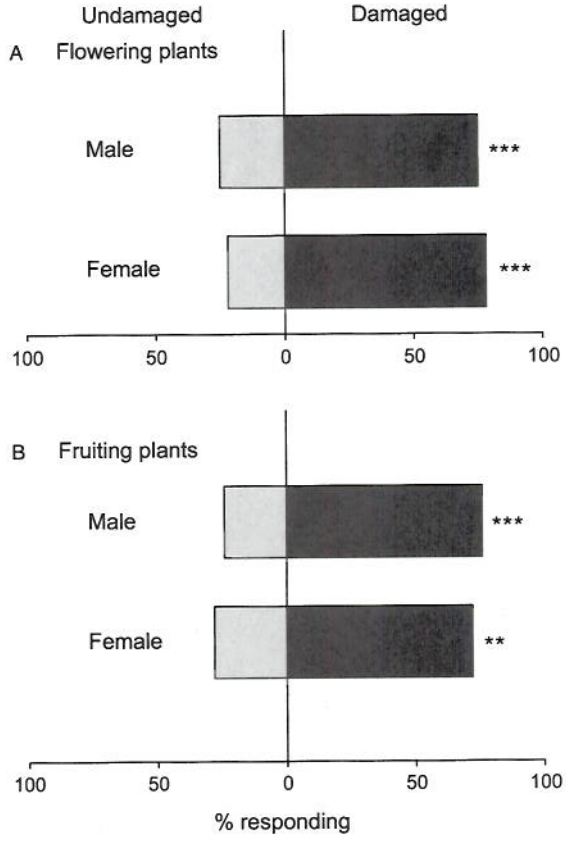

Figure 2 Response of 10-day-old mated *Anthonomus eugenii* to old damage and actively feeding females on (A) flowering and (B) fruiting pepper plants in a Y-tube olfactometer. χ^2: **P<0.01, *** P<0.001; n = 50.

females showed no preference ($\chi^2 = 0.72$, d.f. = 1, P = 0.40; data not shown). In the final assay, there was no difference in the response of male or female weevils to excised pepper fruit with actively feeding weevils over undamaged fruit (male: $\chi^2 = 2.67$, d.f. = 1, P = 0.10; female: $\chi^2 = 0.30$, d.f. = 1, P = 0.59; data not shown). Across all Y-tube assays, an average (± SE) 12.7% ± 2.6 of total males and 11.7% ± 3.1 of total females failed to respond.

Orientation of weevils to induced volatiles in a wind tunnel

There was a greater total upwind response by males to damaged plants than to undamaged plants ($\chi^2 = 6.04$, d.f. = 1, P = 0.014), but females showed no difference in response (Figure 3A). No difference in trap contact between the two treatments was observed for males, however, more females made trap contact in the damaged treatment than in the undamaged treatment ($\chi^2 = 10.51$, d.f. = 1, P = 0.0012; Figure 3C). Equal numbers of males and females made trap contact in the undamaged treatment but more females than males made trap contact in the damaged treatment ($\chi^2 = 6.58$, d.f. = 1, P = 0.010).

In the second wind tunnel experiment (Figure 3B), there was a significantly greater total upwind response for both females ($\chi^2 = 7.06$, d.f. = 1, P = 0.0079) and males ($\chi^2 = 9.55$, d.f. = 1, P = 0.0020) in the active-feeding treatment compared to the damaged treatment, with insects removed. Trap contact was also significantly higher for both males ($\chi^2 = 6.11$, d.f. = 1, P = 0.014) and females ($\chi^2 = 5.07$, d.f. = 1, P = 0.024) in the active-feeding treatment (Figure 3D). Males and females did not differ in upwind response to the damaged or active-feeding treatments (Figure 3B). More males than females made trap contact in the active-feeding treatment ($\chi^2 = 5.89$, d.f. = 1, P = 0.015) and the damaged treatment ($\chi^2 = 4.87$, d.f. = 1, P = 0.027; Figure 3D).

Orientation of weevils in a four-choice olfactometer to headspace volatile collections of fruiting pepper plants

In the preliminary bioassay, both male and female weevils chose headspace volatiles from pepper plants with actively feeding females over air in the Y-tube at a concentration of 0.6 plant h (male: active feeding = 68%: $\chi^2 = 6.48$, d.f. = 1, P = 0.011; female: active feeding = 78%: $\chi^2 = 15.68$, d.f. = 1, P<0.0001; data not shown). In the four-choice assay, females strongly preferred volatiles from damaged plants over volatiles from undamaged plants and solvent control ($\chi^2 = 443.46$, d.f. = 2, P<0.0001), as did males ($\chi^2 = 933.54$, d.f. = 2, P<0.0001; Figure 4A). Both sexes preferred volatiles released from plants with active feeding over volatiles from plants with old damage (female: $\chi^2 = 660.70$, d.f. = 1, P<0.0001; male: $\chi^2 = 690.24$, d.f. = 1, P<0.0001; Figure 4B). Mated female weevils showed no preference between male and female active feeding, but male weevils preferred male active feeding over female active feeding ($\chi^2 = 579.38$, d.f. = 2, P<0.0001; Figure 4C). Unlike mated females, virgin females also preferred the odor of plants with male active feeding over plants with female active feeding ($\chi^2 = 636.46$, d.f. = 2, P<0.0001; Figure 4C). Across all

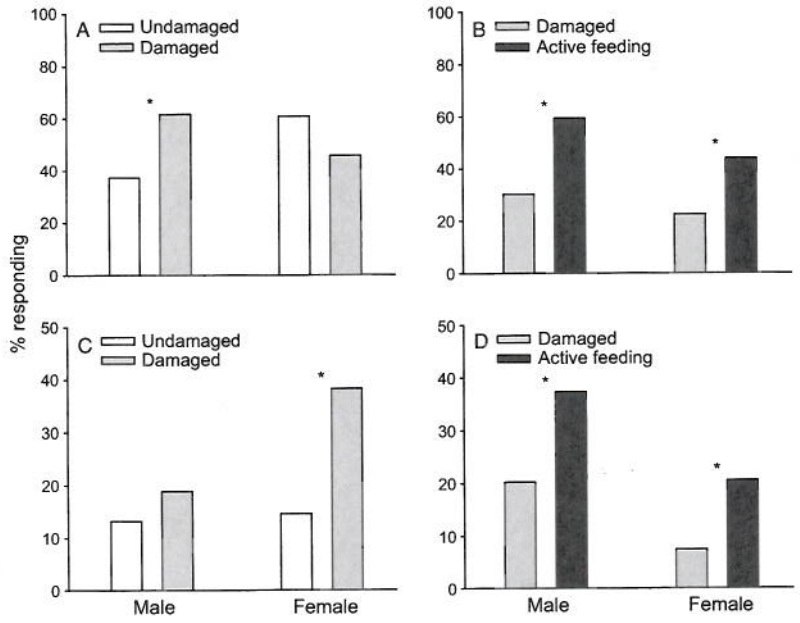

Figure 3 Response of 10-day-old mated *Anthonomus eugenii* to induced volatiles from fruiting pepper plants in no-choice wind tunnel assays. (A) Total upwind response to undamaged and damaged plant treatments, (B) total upwind response to damaged and active feeding plant treatments, (C) trap contact in undamaged and damaged plant treatments, and (D) trap contact in damaged and active feeding plant treatments. Bars labeled with an * are significantly different (χ^2: P<0.05); n = 40.

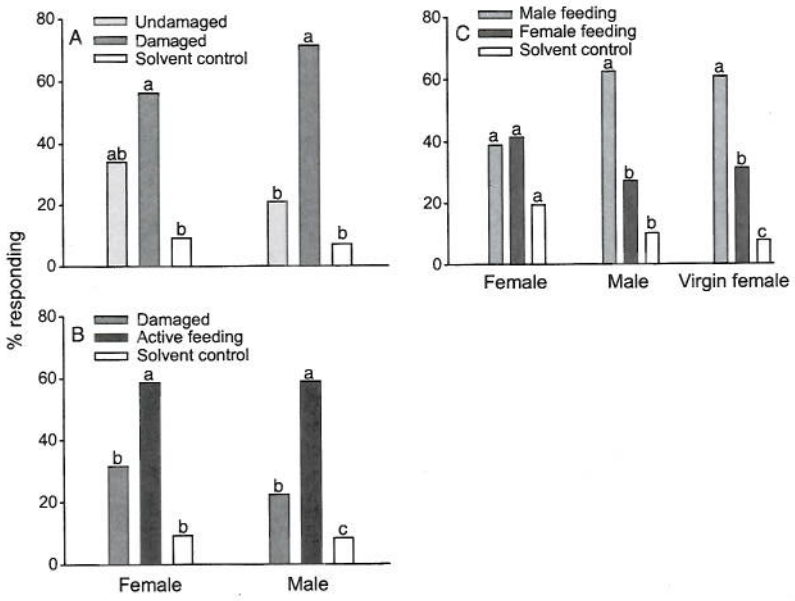

Figure 4 Response of 10-day-old mated and virgin *Anthonomus eugenii* to fruiting pepper plant headspace volatiles in a four-choice olfactometer. Two treatment arms alternated with two solvent control arms. (A) Undamaged vs. female damaged plants, (B) female damaged vs. active feeding, and (C) male feeding vs. female feeding. Bars labeled with different letters are significantly different (χ^2: $P<0.05$); n = 12.

four-arm olfactometer assays, an average (± SE) 32.5% ± 1.4 of total males and 37.1% ± 4.5 of total females failed to respond.

GC-MS analysis of pepper volatiles

Qualitative as well as quantitative differences were observed in volatiles released by undamaged and damaged plants (Figure 5A and B) specifically, an upregulation of Z-3-hexen-1-ol [1] and constitutive volatiles like E-β-ocimene [3], linalool [4], (3E)-4,8-dimethyl–1,3,7-nonatriene [5], (1,3,8-para)-menthatriene [6], methyl salicylate [7], and geranyl-linalool [14]. Most noticeable in the damaged plants was the induced release of several sesquiterpenes (sesquithujene [8], β-elemene [9], (E)-α-bergamotene [10], (E,E)-α-farnesene [11], (E,E)-nerolidol [12]). This change was even more pronounced in plants with active feeding (Figure 6). When the volatile profiles of active feeding damage on fruiting and flowering plants were compared (Figure 6A and B), differences were observed between the plant stages, particularly in the sesquiterpene region (14–24 min) but also by an increased release of methyl salicylate [7] from the fruiting plants. Note that the presence of both β-elemene [9] and the broad baseline drift in the sesquiterpenes area are

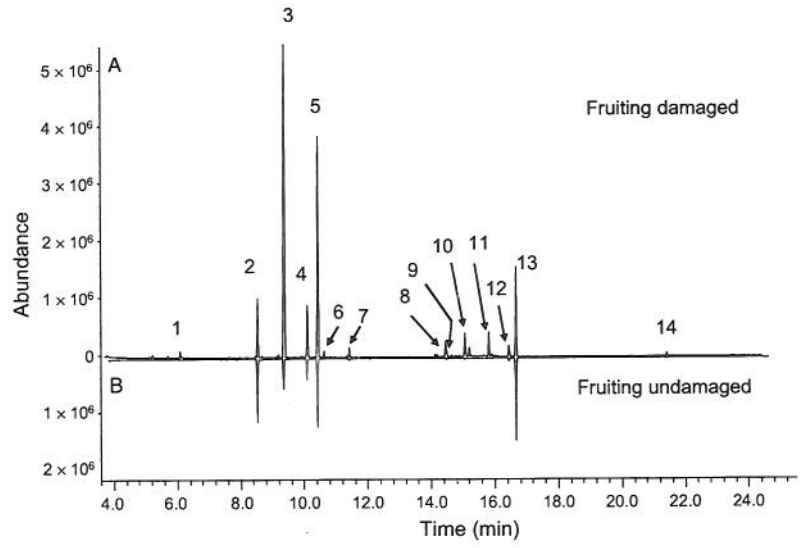

Figure 5 Representative headspace volatile profiles from (A) female *Anthonomus eugenii*-damaged and (B) undamaged fruiting pepper plants. 1. (Z)-3-hexen-1-ol, 2. (Z)-3-hexen-1-yl acetate, 3. (E)-β-ocimene, 4. linalool, 5. (3E)-4,8-dimethyl-1,3,7-nonatriene, 6. (1,3,8-para)-menthatriene, 7. methyl salicylate, 8. sesquithujene, 9. β-elemene, 10. (E)-α-bergamotene, 11. (E,E)-α-farnesene, 12. (E,E)-nerolidol, 13. (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and 14. geranyl-linalool.

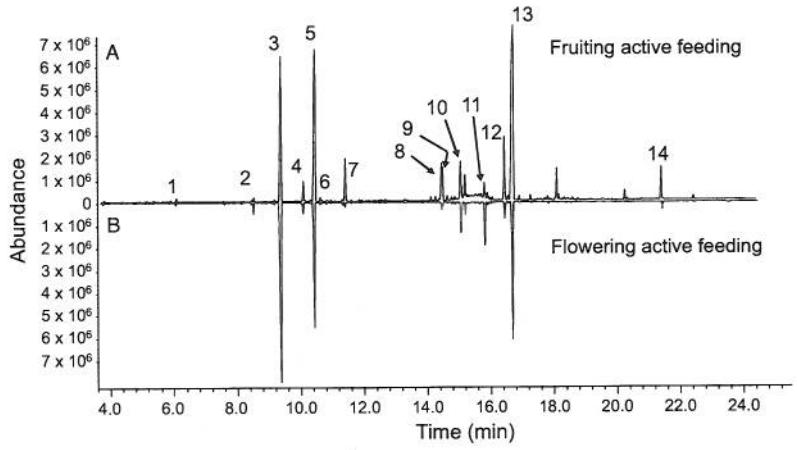

Figure 6 Representative headspace volatile profiles from active female *Anthonomus eugenii* feeding damage on (A) fruiting and (B) flowering pepper plants. 1. (Z)-3-hexen-1-ol, 2. (Z)-3-hexen-1-yl acetate, 3. (E)-β-ocimene, 4. linalool, 5. (3E)-4,8-dimethyl-1,3,7-nonatriene, 6. (1,3,8-para)-menthatriene, 7. methyl salicylate, 8. sesquithujene, 9. β-elemene, 10. (E)-α-bergamotene, 11. (E,E)-α-farnesene, 12. (E,E)-nerolidol, 13. (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and 14. geranyl-linalool.

indications of a thermal degradation of a germacrene type sesquiterpene. On-column GC/MS analyses eliminated the presence β-elemene in our headspace samples, but gave no additional information, thus this terpene remains unidentified.

Males feeding on fruiting plants gave, as expected, a very similar qualitative result to female feeding but with the addition of the six-component male-produced pheromone consisting of (Z)-2-(3,3-dimethyl)cyclohexylidene ethanol, (E)-2-(3,3-dimethyl)cyclohexylidene ethanol, (Z)-(3,3-dimethyl)cyclohexylideneacetaldehyde, (E)-(3,3-dimethyl)cyclohexylideneacetaldehyde, geranic acid, and geraniol (Eller et al., 1994; Figure 7). After 24 h of feeding damage, no change in the qualitative makeup of the excised pepper fruit volatile headspace was observed except for a small increase in constitutive volatiles (spectra not shown).

Discussion

We have previously established that pepper weevil males and females are attracted to constitutive volatiles released by pepper plants (Addesso & McAuslane, 2009) and we hypothesized that plant volatiles act as a major cue for host location. In this paper we addressed the question of how pepper weevil behavior is affected by conspecific feeding damage, the phenological stage of the host plant and the combination of host plant and insect-produced volatiles. The results of our Y-tube, wind tunnel, and four-arm olfactometer assays confirm that pepper weevils prefer damaged over undamaged plants as one would expect if qualitative or quantitative changes in plant volatile emissions made the damaged host more attractive. When damaged plants were tested against those with actively feeding weevils, test weevils preferred plants with active feeding

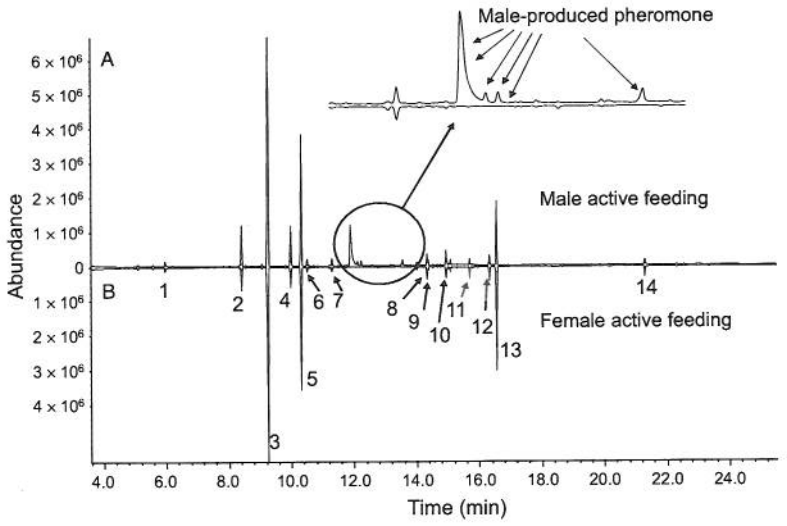

Figure 7 Representative headspace volatile profiles from active feeding of *Anthonomus eugenii* (A) males and (B) females on fruiting pepper plants. 1. (Z)-3-hexen-1-ol, 2. (Z)-3-hexen-1-yl acetate, 3. (E)-β-ocimene, 4. linalool, 5. (3E)-4,8-dimethyl-1,3,7-nonatriene, 6. (1,3,8-para)-menthatriene, 7. methyl salicylate, 8. sesquithujene, 9. β-elemene, 10. (E)-α-bergamotene, 11. (E,E)-α-farnesene, 12. (E,E)-nerolidol, 13. (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and 14. geranyl-linalool.

damage. The attractiveness of active feeding could be due to either a quantitative increase of host plant volatiles emitted from open wounds or from qualitative differences between the volatile profiles of old damage and active feeding damage. Our analysis of volatiles emitted from these two treatments suggests that both explanations are a possibility. In our 1 and 48 h active feeding assays, males showed no preference between the treatments while females were attracted to the plants with 48 h of prior feeding damage. Female preference for the 48 h treatment is most likely the result of a preference for compounds whose induction takes longer than 1 h, such as the various feeding-induced sesquiterpenes. Previous studies by Mechaber (1992) in a Y-tube olfactometer demonstrated that adult cranberry weevils (*A. musculus*) were attracted to damaged cranberry (*Vaccinium macrocarpon* Ait.) flower buds compared with clean air or healthy plants. In a study where blueberry was the host plant, however, male cranberry weevils were repelled by damaged blueberry flower bud volatiles (Szendrei et al., 2009). The vine weevil (*Otiorhynchus sulcatus* F.) preferred weevil-damaged yew and *Euonymus* but not damaged *Rhododendron* or strawberry over air (van Tol et al., 2002). The results of these various studies suggest that behavioral responses of weevils depend on the host plant under investigation so that suggestions of how pepper weevil will respond to feeding damage on other hosts would be speculative at best.

Feeding damage on pepper flowers results in rapid flower abscission, but damaged fruit remain attached far longer and can continue to develop for some time following infestation, making fruit the preferred oviposition site of the pepper weevil. In the Y-tube, we demonstrated that pepper weevils preferred feeding damage on fruiting plants over flowering plants, indicating that the weevil can discriminate between pepper plant stages by their volatile compositions alone, without the need for visual or gustatory cues. The analysis of flowering and fruiting plant volatiles confirms differences in volatile composition between plant treatments. This change in preference for different phenological stages of a plant has already been demonstrated for cranberry weevil on blueberry, where females showed a distinct preference for open over closed flower buds (Szendrei et al., 2009). Kalinova et al. (2000) also suggested that phenological differences in bud volatile emissions within cultivars may play a role in host searching behavior of the apple blossom weevil upon noting that volatile plumes of buds from two cultivars differed both between cultivars and across four phenological stages.

In addition to host plant volatiles, pepper weevils may also use pheromone when locating host plants. The presence of the male weevil's aggregation pheromone alongside induced plant volatiles has the potential to affect males and females differently. In the case of the pepper weevil, field tests demonstrated that both male and female weevils are attracted to aggregation pheromone baited traps (Coudriet & Kishaba, 1988; Bottenberg & Lingren, 1998). In our plant bioassays, we focused on 10-day-old mated adults. These males demonstrated only a slight but non-significant preference for the male feeding treatment while females showed no preference. This was not what we expected, given the results of the previous aggregation pheromone tests where both males and females were attracted to pheromone traps (Coudriet & Kishaba, 1988; Bottenberg & Lingren, 1998). Our results led us to suspect that pheromone production was uneven across the replicates. We therefore repeated the experiment using headspace collections (Figure 4) in which we were able to confirm the presence of the aggregation pheromone components by GC-MS prior to presenting the volatiles on filter paper in a four-choice bioassay. We also tested virgin females of the same age in order to determine whether mating status was a factor in female response. While mated females showed no preference for the two treatments, males and virgin females preferred the headspace volatiles released by male feeding in the four-arm olfactometer. Our results in the headspace assay supported previous field observations and added information about the effect of mating status on female response. The shift in female response is expected if an unmated female's primary goal is to locate a mate, while mated females are searching for oviposition sites.

Studies of the boll weevil have shown that the combination of plant and insect-derived volatiles are more attractive than either alone. Similarly, field traps containing a combination of pheromone component (grandisoic acid) and benzaldehyde caught more plum curculios than traps baited with pheromone or benzaldehyde alone (Pinero & Prokopy, 2003). Recent studies of several insects demonstrated positive responses to plant/pheromone combinations including the codling moth (Yang et al., 2004), old house borer (Reddy et al., 2005), oriental fruit moth (Pinero & Dorn, 2007), Colorado potato beetle (Dickens, 2006), and the Asian longhorned beetle (Nehme et al., 2010). The combination of pheromone and host plant volatiles for pepper weevil attraction may be particularly important given that it shares pheromone components (grandlure II, III, and IV) with its congeners the boll weevil (Tumlinson et al., 1969) and strawberry blossom weevil (Innocenzi et al., 2001), as well as the pecan weevil, *Curculio caryae* (Horn) (Hedin et al., 1997).

A commercial pepper weevil trap comprised of a combination of yellow sticky trap and aggregation pheromone lure (Pherocon; Trécé Inc, Adair, OK, USA) is currently available. Our results suggest that for males and virgin

females, a combination of plant volatiles and pheromone would be a more effective monitoring tool while monitoring of gravid females might require a plant-based lure if they avoid the pheromone in the field. Pepper weevil responded in the same manner to headspace collections of plant volatiles as they did to whole plant treatments, indicating the method is sufficient for the collection of attractive compounds. Electrophysiological and behavioral bioassays are currently underway to identify attractive compounds emitted from active feeding treatments. The goal is to formulate a plant volatile blend that can be used to increase the effectiveness of the current pepper weevil monitoring system.

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References

Addesso KA & McAuslane HJ (2009) Pepper weevil attraction to volatiles from host and non-host plants. Environmental Entomology 38: 216–224.

Bichao H, Borg-Karlson AK, Wibe A, Araujo J & Mustaparta H (2005a) Molecular receptive ranges of olfactory receptor neurones responding selectively to terpenoids, aliphatic green leaf volatiles and aromatic compounds, in the strawberry blossom weevil *Anthonomus rubi*. Chemoecology 15: 211–226.

Bichao H, Borg-Karlson AK, Araujo J & Mustaparta H (2005b) Five types of olfactory receptor neurons in the strawberry blossom weevil *Anthonomus rubi*: Selective responses to inducible host-plant volatiles. Chemical Senses 30: 153–170.

Bottenberg H & Lingren B (1998) Field performance of a new pepper weevil pheromone formulation. Proceedings of the Florida State Horticultural Society 111: 48–50.

Coudriet DL & Kishaba AN (1988) Bioassay procedure for an attractant of the pepper weevil (Coleoptera: Curculionidae). Journal of Economic Entomology 81: 1499–1502.

Dickens JC (1984) Olfaction in the boll weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae): Electroantennogram studies. Journal of Chemical Ecology 10: 1759–1785.

Dickens JC (1986) Orientation of boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), to pheromone and volatile host compound in the laboratory. Journal of Chemical Ecology 12: 91–98.

Dickens JC (1989) Green leaf volatiles enhance aggregation pheromone of boll weevil, *Anthonomus grandis*. Entomologia Experimentalis et Applicata 52: 191–203.

Dickens JC (2006) Plant volatiles moderate response to aggregation pheromone in Colorado potato beetle. Journal of Applied Entomology 130: 26–31.

Dickens JC & Moorman EE (1990) Maturation and maintenance of electroantennogram responses to pheromone and host odors in boll weevils, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) fed their host plant or an artificial diet. Journal of Applied Entomology 109: 470–480.

Dickens JC, Jang EB, Light DM & Alford AR (1990) Enhancement of insect pheromone responses by green leaf volatiles. Naturwissenschaften 77: 29–31.

Eller FJ (1995) A previously unknown sexual character for the pepper weevil (Coleoptera: Curculionidae). Florida Entomologist 78: 180–183.

Eller FJ, Bartelt RJ, Shasha BS, Schuster DJ, Riley DG et al. (1994) Aggregation pheromone for the pepper weevil *Anthonomis eugenii* Cano (Coleoptera: Curculionidae): identification of field activity. Journal of Chemical Ecology 20: 1537–1555.

Genung WG & Ozaki HY (1972) Pepper Weevil on the Florida East Coast. EREC Belle Glade Mimeo Report, EV-2, University of Florida, Gainesville, FL, USA.

Hedin PA, Mckibben GH, Mitchell EB & Johnson WL (1979) Identification and field evaluation of the compounds comprising the sex pheromone of the female boll weevil. Journal of Chemical Ecology 5: 1573–1561.

Hedin PA, Dollar DA, Collins JK, Dubois JG, Mulder PG et al. (1997) Identification of male pecan weevil (*Curculio caryae*) pheromone. Journal of Chemical Ecology 23: 965–977.

Innocenzi PJ, Hall DR & Cross JV (2001) Components of male aggregation pheromone of strawberry blossom weevil, *Anthonomus rubi* Herbst. (Coleoptera: Curculionidae). Journal of Chemical Ecology 27: 1203–1217.

Kalberer NM, Turlings TCJ & Rahier M (2001) Attraction of a leaf beetle to damaged host plants. Journal of Chemical Ecology 27: 647–661.

Kalinova B, Stransky K, Harmatha J, Ctvrtecka R & Zd'arek J (2000) Can chemical cues from blossom buds influence cultivar preference in the apple blossom weevil (*Anthonomus pomorum*)? Entomologia Experimentalis et Applicata 95: 47–52.

Landolt PJ & Phillips TW (1997) Host plant influences on sex pheromone behavior of phytophagous insects. Annual Review of Entomology 42: 371–391.

Loughrin JH, Potter DA, Hamilton-Kemp TR & Byers ME (1996) Role of feeding-induced plant volatiles in aggregative behavior of the Japanese beetle (Coleoptera: Scarabaeidae). Environmental Entomology 25: 1188–1191.

Mechaber WL (1992) Ecology of *Anthonomus musculus*: Host Plant Finding and Exploitation by Cranberry Weevil. PhD Dissertation, Department of Biology, Tufts University, Medfrod, MA, USA.

Meiners T, Hacker NK, Anderson P & Hilker M (2005) Response of the elm leaf beetle to host plants induced by oviposition and feeding: the infestation rate matters. Entomologia Experimentalis et Applicata 115: 171–177.

Nehme ME, Keena MA, Zhang A, Baker TC, Xu Z & Hoover K (2010) Evaluating the use of male-produced pheromone components and plant volatiles in two trap designs to monitor

Anoplophora glabripennis. Environmental Entomology 39: 169–176.

Paré PW & Tumlinson JH (1999) Plant volatiles as a defense against insect herbivores. Plant Physiology 121: 325–331.

Patrock RJ & Schuster DJ (1987) Field survey for the pepper weevil, *Anthonomus eugenii*, on nightshade. Proceedings of the Florida Horticultural Society 100: 217–220.

Patrock RJ & Schuster DJ (1992) Feeding, oviposition, and development of the pepper weevil (*Anthonomis eugenii* Cano), on selected species of Solanaceae. Tropical Pest Management 38: 65–69.

Pinero JC & Dorn S (2007) Synergism between aromatic compounds and green leaf volatiles derived from the host plant underlies female attraction in the oriental fruit moth. Entomologia Experimentalis et Applicata 125: 185–194.

Pinero JC & Prokopy RJ (2003) Field evaluation of plant odor and pheromonal combinations for attracting plum curculios. Journal of Chemical Ecology 29: 2735–2748.

Reddy GVP & Guerrero A (2004) Interactions of insect pheromones and plant semiochemicals. Trends in Plant Science 9: 253–261.

Reddy GVP, Fettkother R, Noldt U & Dettner K (2005) Enhancement of attraction and trap catches of the old-house borer, *Hylotrupes bajulus* (Coleoptera: Cerambycidae), by combination of male sex pheromone and monoterpenes. Pest Management Science 61: 699–704.

SAS Institute, Inc. (2006) SAS, version 9.1. SAS Institute, Cary, NC, USA.

Szendrei Z, Malo E, Stelinski L & Rodriguez-Saona C (2009) Response of cranberry weevil (Coleoptera: Curculionidae) to host plant volatiles. Environmental Entomology 38: 861–869.

van Tol RWHM, Visser JH & Sabelis MW (2002) Olfactory responses of the vine weevil, *Otiorhynchus sulcatus*, to tree odors. Physiological Entomology 27: 213–222.

Tumlinson JH, Hardee DD, Gueldner RC, Thompson AC, Hedin PA & Minyard JP (1969) Sex pheromones produced by male boll weevils: isolation, identification and synthesis. Science 166: 1010–1012.

Yang ZH, Bengtsson M & Witzgall P (2004) Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella*. Journal of Chemical Ecology 30: 619–629.